FORM PTO-1390 (Modified) (REV 11-2000) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER	U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR)
18111-001NATL	09/868748

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/IB99/02020	December 16, 1999 (16/12/99)	December 21, 1998 (21/12/98)

TITLE OF INVENTION

Function-Based Small Molecular Weight Compound Screening System In Drosophila Melanogaster

APPLICANT(S) FOR DO/EO/US

HAFEN, Ernst

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include itens (5), (6), (9) and (24) indicated below.
4. ☐ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
   a. ☒ is attached hereto (required only if not communicated by the International Bureau).
   b. ☒ has been communicated by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. ☐ is attached hereto.
   b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
   a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   b. ☐ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
10. ☐ An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).
11. ☐ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☒ A copy of the International Search Report (PCT/ISA/210).

Items 13 to 20 below concern document(s) or information included:

13. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☐ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☐ A substitute specification.
18. ☐ A change of power of attorney and/or address letter.
19. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
20. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
21. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
22. ☒ Certificate of Mailing by Express Mail
23. ☒ Other items or information:

Return Receipt Postcard;
Express Mail Label No.: EK555671582US
Date of Deposit: June 20, 2001

LICATION NO. (IF KNOWN, SEE 37 CFR) 09/868748	INTERNATIONAL APPLICATION NO. PCT/IB99/02020	ATTORNEY'S DOCKET NUMBER 18111-001NATL

24. The following fees are submitted:.

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :

		CALCULATIONS PTO USE ONLY
☐	Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO	**$1000.00**
☒	International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO	**$860.00**
☐	International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO	**$710.00**
☐	International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4)	**$690.00**
☐	International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4)	**$100.00**

ENTER APPROPRIATE BASIC FEE AMOUNT =	**$860.00**	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☒ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).	**$130.00**	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	17 - 20 =	0	x $18.00	**$0.00**	
Independent claims	3 - 3 =	0	x $80.00	**$0.00**	
Multiple Dependent Claims **(check if applicable)**.			☐	**$0.00**	
TOTAL OF ABOVE CALCULATIONS =				**$990.00**	
☒ Applicant claims small entity status. (See 37 CFR 1.27). The fees indicated above are reduced by 1/2.				**$495.00**	
SUBTOTAL =				**$495.00**	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (f)). +				**$0.00**	
TOTAL NATIONAL FEE =				**$495.00**	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) **(check if applicable).** ☐				**$0.00**	
TOTAL FEES ENCLOSED =				**$495.00**	
				Amount to be: refunded	$
				charged	$

a. ☒ A check in the amount of **$495.00** to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. _____ in the amount of _____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. **50-0311** A duplicate copy of this sheet is enclosed.

d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

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FUNCTION-BASED SMALL MOLECULAR WEIGHT COMPOUND SCREENING SYSTEM IN *DROSOPHILA MELANOGASTER*

FIELD OF THE INVENTION

The present invention relates to the use of genetically-modified strains of *Drosophila melanogaster* in high-throughput screening (HTS) of small molecular weight compounds. The present invention also relates to the systematic identification of small molecules which interfere with specific disease pathways using *Drosophila* as the genetic model system. Additionally, the present invention relates to the use of automated screening systems employing microinjection of small molecular weight compounds, possessing putative biological activity, into the open circulatory system (i.e., the hemolymph) of genetically-modified *Drosophila* larva.

BACKGROUND OF THE INVENTION

The identification and subsequent characterization of new therapeutics are the primary rate limiting step in pharmacological research. Pharmacological compounds have been sought from natural products for many years. In general, complex mixtures derived from cells, or their secondary metabolites, are screened for biological activity. Subsequently, when the desired biological activity is identified in such a complex mixture, the specific molecule which possesses the activity has been purified, using the biological activity as the means for identifying the component of the mixture which possesses the desired biological activity.

Many of the existing therapeutics on the market to date have been identified in an accidental manner, and frequently their mechanism of action is poorly understood. A more direct approach towards the identification of new small molecular weight compounds effective against various disease conditions requires some knowledge of both the molecular defect underlying a given disease and of the cellular pathways and processes in which the defective component is acting. In fact, such knowledge of the pathways involved is essential since the defective gene product may not be the best target for a small molecular weight compound. In addition, despite the great value that large libraries of molecules can have for identifying useful compounds or improving the properties of a lead compound, the difficulties of screening such libraries, particularly extremely large libraries, has limited the impact that access to such

libraries should have made in reducing the costs of drug discovery and development. This is, in a large part, due to the weaknesses inherent in the current screening methodologies of compound libraries which employ both cell-free and *in vitro* cell-based assay systems.

Accordingly, there remains an as yet unfulfilled need within the relevant fields for a rapid, quantitative HTS screening methodology that may be fully automated and that utilizes a genetic model possessing, but not be limited to, the following characteristics: (i) a high degree of conservation of the various signaling pathways involved in the etiology of disease, especially human disease; (ii) the ability to be grown rapidly in large numbers with little effort; (iii) a stable genetic mutation(s) and (iv) an easily discernible genetic outcome for use in the screening procedure.

SUMMARY OF THE INVENTION

The present invention targets the ubiquitous nature of the biochemical pathways present in most every cell and couples them to an assay system of choice within the appropriately modified *Drosophila* strain. More specifically, the present invention utilizes genetically sensitized *Drosophila* strains that possess mutations within a selected biochemical pathway in the construction of a high-throughput screen (HTS) of small molecular weight compounds to facilitate the identification and characterization of novel, lead drug candidates that dominantly modify the phenotypic effects of the chosen sensitized *Drosophila* biochemical pathways.

The present invention discloses a methodology for the screening of compounds for desirable biological/therapeutic activities that involves the screening of individual chemical compounds which have been synthesized and cataloged in libraries of drug or chemical companies or research institutes. The active "lead" compounds and novel chemical entities identified and characterized by the present invention may be utilized for the development of bioactive "leads" in small molecule libraries for pharmaceuticals, agrochemicals and the like.

The high degree of conservation of morphogenetic processes between *Drosophila* and humans has made *Drosophila* a prime model system for the identification of new putative drug targets using function-based genetic approaches. Genetically sensitized *Drosophila* systems, wherein the gene modification results in a dose-sensitive phenotype, permit detection of a mere two-fold effect of small compounds on specific signaling pathways related to human diseases. Such *Drosophila* strains may be genotypic (+/null), and hence are hemizygous for a dose sensitive gene within a given biochemical pathway.

The present invention is a novel combination of an automation-capable, *in vitro* HTS assay with an *in vivo* "readout" system comprised of *Drosophila melanogaster* that are genetically-sensitized for a specific chosen biochemical pathway, preferably a human disease pathway. Hence, the present invention discloses a methodology that serves to "bridge the gap" of the presently-utilized screening systems by use of a well-established model for human disease (*Drosophila*) for the screening of large numbers of compounds in a rapid, quantitative and highly efficacious manner. The expression of human disease genes or their homologs within any chosen stage of the developing *Drosophila* larva should model the effects of these genes in human cells and subsequently produce phenotypes that are modified by either the mutations within these interacting genes, or by compounds that block the function of the corresponding gene product. These gene products are prime candidates as targets for small compounds which interfere with their function.

The preferred embodiment of the present invention employs an automation-capable system for the microinjection of compound(s) of interest into the open circulatory system (i.e., hemolymph) of *Drosophila* larvae, in particular the *Drosophila* first, second, or third instar larvae, that have been previously genetically-modified in a gene that is specific to a signaling pathway involved in human disease. The modified gene may be involved in any biochemical pathway, such as the Ras signaling pathway, and have a functional phenotype that is readily scorable, such as by observation of the developing *Drosophila* eye.

The invention may use any biochemical pathway to monitor phenotypic changes involved in the development of any imaginal disk. Therefore, any stage of Drosophila larval development may be used in the analysis. Following maturation of the microinjected *Drosophila* larvae, the biological effect of the injected compound(s) are assessed, preferably in adult flies. In addition, the screening methodology disclosed by the present invention may allow the simultaneous observation of: (i) the general biological toxicity of the microinjected compound(s) through 50% lethal dose (LD50) computations: (ii) the specificity of the modification of the specific *Drosophila* phenotype (i.e., suppression of the rough eye phenotype) and (iii) the non-specific interference with other well-defined developmental and physiological pathways.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can

be used in the practice or testing of the present invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In the case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

Other features and advantages of the invention will be apparent from the following detailed description and claims.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a series of scanning electron micrographs of the *Drosophila* compound eye (Panels A-D) and light micrographs of tangential cross-sections of the *Drosophila* compound eye (Panels E-H) following genetic modification of the Raf^{torY9} phenotype by *Su*(*Raf*) and two E(*Raf*) mutations. Panels A and E illustrate the wild-type genotype; Panels B and F illustrate the Raf^{torY9}/+ genotype; Panels C and G illustrate the Raf^{torY9}/+:*Su*(*Raf*)$3A^{9J1}$ genotype and Panels D and H illustrate the Raf^{torY9}/+ , +/E(*Raf*)$2A^{16T1}$ genotype. It should be noted that the *Su*(*Raf*) and two E(*Raf*) mutations dominantly-modify the overall degree of *Drosophila* compound eye texture roughening in Raf^{torY9} flies. In contrast, in the case of *Su*(*Raf*)3A, all ectopic R7 cells were eliminated and the eye regained the smooth texture observed in wild-type Drosophila.

FIG. 2 is a schematic illustration of the known or estimated cytological chromosomal locations of all six *Su*(*Raf*) and two E(*Raf*) complementation groups (loci). All loci were mapped to either chromosome 2 or 3. In addition, the approximate chromosomal breakpoints of selected deficiencies are also shown. Deficiencies that both modified the Raf^{torY9} phenotype and failed to complement EMS-induced *Su*(*Raf*) and two E(*Raf*) mutations are indicated by the solid black box (■). The two deficiencies that failed to complement *Su*(*Raf*)3A but did not appreciably suppress Raf^{torY9} are indicated by the lightly-shaded box (□). The deficiencies completely spanning the interval to which *Su*(*Raf*)3A was mapped, but that failed to modify either the Raf^{torY9} phenotype or display an obvious phenotype in trans to *Su*(*Raf*)$3A^{18A2}$, are indicated by the non-shaded box (□).

FIG. 3 is a graphic illustration of the survival rates of third instar R10 larvae injected with 10 μM rapamycin (dark bars) or a control solution (light bars).

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides an efficient methodology for the quantitative, systematic identification of small molecular weight molecules which interfere with specific disease conditions in an animal model system. Genetically-modified stains of the fruitfly, *Drosophila melanogaster*, have played a pivotal role in the identification of new genes whose products act in signaling pathways that are altered in human diseases (see e.g., Dickson, et al., 1996. Genetics 142:163-171; Dominguez, et al., 1996. Dev. Biol. 7:219-226). Such genetically sensitive backgrounds have been used previously to identify mutations in genes coding for rate limiting components in a particular signaling pathway. The present invention makes use of such genetically-modified *Drosophila* strains to screen small molecular weight compounds of interest for biological activity which functions to interfere with specific disease-related pathways.

Currently, numerous drug screening protocols rely upon high-throughput screening (HTS) of compound libraries using cell-free or *in vitro* cell-based assay systems. HTS is a process by which large numbers of compounds with putative biological activity may be tested, for example in an automated manner, for activity as inhibitors (antagonists) or activators (agonists) of a specific biological target (e.g., cell-surface receptor or a metabolic enzyme). However, current HTS methods possess a number of limitations such as: (i) bioavailability; (ii) pharmacokinetics; (iii) toxicity and (iv) absolute specificity. Hence, subsequent medicinal chemical and pharmacological studies are required to convert a compound which emerges from an initial HTS screening into a therapeutically useful drug.

The potent and specific biological activities of many low molecular weight peptides make these molecules attractive starting points for therapeutic drug discovery (see e.g., Hirschmann, et al., 1991. Angew. Chem. Int. Ed. Engl. 30:1278-1301). The present invention discloses a methodology for the screening of compounds for desirable biological/therapeutic activities that involves the screening of individual chemical compounds that have been synthesized and cataloged in libraries of drug or chemical companies or research institutes. The active "lead" compounds and novel chemical entities identified and characterized by the present invention may be any chemical compound of interest, and may be utilized for the development of bioactive "leads" in small molecule libraries for pharmaceuticals, agrochemicals and the like.

With respect to the generation of small molecular weight compound libraries of the present invention, the combination of biochemical diversity is often synergistic with the metabolic diversity obtained from the *in vivo* production of "natural products". Collections of naturally or synthetically produced chemical or oligomeric compounds, for example peptides, can be administered to cultures of microorganisms. In accord, each microbial strain may potentially create numerous modified chemical derivatives, thus generating a "metabolite library". Because each of these aforementioned cultures would (potentially) contain a very complex mixture of metabolites, a highly efficacious method of screening would be required (i.e., HTS). An aliquot of the library is incubated with each of the many strains typical of a microorganism fermentation screening program, and the media screened utilizing an HTS-based assay. In another aspect of the invention, natural product diversity is screened by creating a mixture of combinatorially-tagged liposomes; wherein each liposome encapsulates, for example, only one member or a simple mixture of a natural product compound library. The libraries which are generated by the methodologies disclosed herein may be screened for any biological activity known within the art. These include, but are not limited to: anti-microbial activity, anti-tumor activity, enzyme inhibiting activity, receptor binding, growth promotion activity, and *in vitro* and *in vivo* tests for biological responses.

With the advent of genomics, combinatorial paradigms and high-throughput screening (HTS) assay-based pharmacological testing, the number of compounds possessing biological/therapeutic activity is likely to markedly increase. HTS assays are, preferably, based upon automation, validation and integration of *in vitro* absorption-metabolism models and database management (see e.g., Rodrigues, et al., 1997. Pharm. Res. 14:1504-1510). Complementary to this tenet is the need to generate a taxonomy of known compounds, identifying those with similar mechanisms, preferably in a way that provides clues as to the nature of those mechanism.

The present invention discloses a screening methodology that is based upon the utilization of genetically-sensitized *Drosophila* as a model system with a new method for drug administration that permits both high-throughput and automation. Based upon previous experience with genetic screens for dominant modifier mutations, genetically-sensitized systems comprising specific pathways that are sensitive to such modifier mutations will permit detection of mere two-fold effects of small molecular weight compounds on specific signaling pathways related to human diseases. This assay sensitivity results from the ability to

differentiate dose-dependent phenotypes of the modified *Drosophila* related to the presence of none, one, or two modified alleles within its genome. The methodology disclosed by the present invention is both more powerful and more sensitive than traditional, cell-free or *in vitro* cell-based assay systems. In addition, the *Drosophila*-based screening assay disclosed herein is based upon a biological "readout" related to human disease, and its intrinsic ability to test for both the overall specificity and toxicity of a compound(s) of interest.

Utilization of *Drosophila melanogaster* as a Model System for the Genetic Dissection of Cellular Processes

The fruitfly, *Drosophila melanogaster*, has been used as an ubiquitous model for the characterization of cellular processes (e.g., signaling pathways) involved in a variety of human diseases. A high degree of conservation of morphogenetic processes between *Drosophila* and humans has made *Drosophila* a prime model system for the identification of putative drug targets using function based genetic approaches.

Drosophila eye development is a prime example of a model system for the study of well defined and functionally integrated genetic controls, and it has been used extensively for the systematic genetic dissection of conserved signaling pathways. The *Drosophila* compound eye is composed of a hexagonal array of approximately 800 identical units, called ommatidia. Each of these units consists of eight photoreceptor cells (R1-R8), four lens-secreting cone cells, and pigment cells that optically insulate each ommatidium. This highly organized structure develops from a single layer epithelial sheet, the eye imaginal disc, during larval and pupal stages by the stepwise recruitment of cells into the ommatidial clusters. During this process, differentiating cells specify the fate of neighboring, but still undetermined, cells by inductive signals.

Drosophila eye development has provided most of the important genetic information regarding evolutionarily conserved mechanisms. While the concept of wide-ranging evolutionary gene conservation is not new, it has been critical in elucidating many complexities in, for example, the Ras and tyrosine kinase signaling pathways via the analysis of *Drosophila* eye mutants. These genes encode components of a highly conserved signaling cascade that has in part been described in vertebrate cells. Activation of the sev (*sevenless*) receptor by boss (*bride of sevenless*) protein presumably results in receptor dimerization and subsequent autophosphorylation on tyrosine residues. This autophosphorylation creates binding sites for

the Drk SH2/ SH3 adaptor protein (see e.g., Raabe, et al., 1995. EMBO J 14:2509-2518). Drk binds to sev via its SH2 domain and to the C-terminus of Sos (*son of sevenless*) protein via its SH3 domains, and thereby brings the Sos protein to the membrane. Sos is a guanine nucleotide releasing factor that activates Ras1 protein by facilitating the conversion from GDP-Ras1 to GTP-Ras1. Ras1 in turn activates a cascade of three cytoplasmic kinases, namely, the homologs of Raf, MEK (MAPK kinase) and MAP kinase encoded by the genes *raf, Dsor1,* and *rolled,* respectively (Dickson, et al., 1996 Genetics 142:163-171). All of these cytoplasmic components are also required for signaling by other RTKs in *Drosophila,* including both torso and DER (see e.g., Chang, et al., 1994. Cold Spring Harbor Symp. Quant. Biol. 59:219-226; Dominguez & Hafen, 1996. Drosophila Sem. Cell Dev. Biol. 7:219-226). The elucidation of these signaling pathways are of great importance as they control various aspects of human developmental regulation, hormone action, and neoplasia.

Genetically manipulated *Drosophila* have served as a powerful tool for dissecting and characterizing gene pathways. Recombinant methodologies used to modify specific *Drosophila* genes are well known in the art, as are methodologies used for the maintenance of said *Drosophila* strains, said strains being homozygous, hemizygous, or heterozygous for defined allelic combinations of the gene or genes of interest. One such phenotype is an irregular, rough eye surface induced upon activation of a genetically modified *raf* gene in the Ras signaling pathway. In such a genetically sensitized background, mutations in genes coding for rate limiting components in a particular biochemical and/or signaling pathway can be identified as modifiers of the phenotype (see e.g., Dickson, et al., 1996. Genetics 142:163-171). These mutations thus identify gene products whose activity or function is critical for the development the disease-related phenotype.

A variety of cell-fate decisions are controlled by the activation of receptor tyrosine kinases (RTKs). One such interaction involves the Drosophila rough eye phenotype which is an irregular, rough eye surface induced upon activation of a genetically-modified *raf* gene (a downstream effector of the *Ras1* oncogene) in the Ras signaling pathway (see e.g., Dickson, et al., 1996. Genetics 142:163-171). In such a genetically-sensitized background, mutations in genes encoding rate limiting components in a particular signaling pathway may be identified as modifiers of the phenotype. These mutations thus identify gene products whose activity or function is critical for the development the disease-related phenotype.

The formation of R7 photoreceptor cells is a model system where scientists have a complete knowledge of all genes acting in the signal transmission pathway that extends from the cell membrane to the nucleus (see e.g., Dominguez, et al., 1997. Dev. Biol. 7:219-226). Although extensive genetic screens for recessive, viable mutations affecting the development of the R7 cells have been carried out, mutations in only four genes apart from *sev* and *boss* have been identified. Flies homozygous for mutations in the *sina* gene (which encodes a nuclear protein) lack R7 cells and are non-viable. Mutations in *Gap1* (encoding a homolog of a GTPase activating protein), *yan* (encoding a transcription factor of the ETS-family) and *tramtrack* (*ttk*) have been shown to cause the formation of multiple R7 cells in each ommatidium (see e.g., Dominguez, et al., 1997. Dev. Biol. 7:219-226). These results tends to suggest that *Gap1*, *yan* and *ttk* act as inhibitors of the R7 specification; whereas *sina* acts as an activator.

Mutations in genes whose products are also involved in other developmental decisions prior to the formation of the eye may cause less informative phenotypes such as lethality. Using current methodologies, their role in R7 development cannot be tested directly and alternative genetic strategies are thus required to identify them. The most successful approach to date utilizes a hypomorphic *sev* mutation that encoded a partially functional, temperature-sensitive *sev* receptor that, at an intermediate temperature, provides barely sufficient activity to specify R7 cells. See, Simon et al., 1991, Cell 67:701-716. In this sensitized genetic background, normally recessive mutations acted dominantly in the R7 decision. A similar system utilizing the multiple R7 phenotypes in *sev* gain of function (GOF) mutations (sev^{GOF}) determines the overall number of cells of the R7 equivalence group which assume R7 cell fate, and so provides a sensitive measure of the *sev* kinase activity and signal transduction efficiency. The recruitment of extra R7 cells disrupts the hexagonal array of the ommatidial units and causes a roughening of the external surface of the eye. Mutations that reduce the efficiency of *sev* signaling are thus detected as suppressors of the rough eye phenotype of sev^{G0F27}.

The value of *Drosophila* as a screening system for evaluating the biological activities of chemicals has been well-documented (see e.g., Schulz, et al., 1955. Cancer Res. 3 (suppl.): 86-100; Schuler, et al., 1982. Terat. Carcin. Mutag. 2:293-301). Small numbers of chemical substances are administered to larvae or flies by feeding, and flies are then analyzed for survival and for phenotypic alterations. These conventional tests do not permit high-throughput screens, nor permit a directed search for small molecular weight compounds that

interfere with a specific morphogenetic pathway related to a human disease condition. Application of compounds by feeding requires relatively large amounts of the substance, and its uptake by the larvae and thus its final concentration is, at best, difficult to control. Furthermore, application by feeding does not permit automation of the procedure necessary for high-throughput analysis.

Use of Drosophila in the present invention

It is contemplated that any biochemical pathway may be utilized in the present invention, including but not limited to metabolic and signaling pathways. Many, if not all, metabolic and signaling pathways are present in every living cell. However, only a subset of pathways are active at any given time during cellular development and differentiation. The present invention would deliver small molecule compounds to be tested at the development stage when the activity of the desired pathway is required. Any stage in *Drosophila* development is contemplated, including but not limited to all larval stages such as the first, second and third instar larval stage of development. In *Drosophila*, the most sensitive phenotypic changes occur in the developing imaginal disks in the third instar larva, as most differentiation occurs at this stage.

Metabolic and signaling pathways, which play important roles in human diseases, may be the "targets" of this screening assay. Cell specificity is conferred upon activation of a specific signaling pathway by effector genes located downstream of a given biochemical pathway. It is these downstream effector genes that determines the cell- or tissue-specific phenotype, even though the signaling pathway may actually be indigenous to all cells. These signaling pathways may utilized intracellular "signals" that are normally turned on within the cells of interest, or they may utilize ectopic pathways. Ectopic expression of a component of a given pathway may be induced during development in either a temporally or spatially restricted manner. If the intact signaling pathway to be targeted is present within a cell which ectopically expresses the induced component, the component will activate the targeted signal transduction pathway. The prime target of a signaling pathway is ideally the component which is the most sensitive to alterations within the signaling pathway. The assay methodology of the present invention relies upon the screening of small molecular weight compounds to identify lead compounds that modify essential components in a given signaling pathway, resulting in an observably altered phenotype in comparison to untreated, wild-type Drosophila.

One experimental approach for the identification of new components of any given biological process is to search for mutations which dominantly modify the effects of another mutation disrupting the same biological process. This technique allows these aforementioned mutations to be recovered in a simple one-generation (F_1) screening assay. More importantly, by sensitizing only a single biological pathway, one may create conditions in which even a slight reduction of gene activity (e.g., due to the loss of only one functional copy of the gene) can result in a detectable phenotype. This is of particular utility when the gene is involved in many other cellular processes, and a more severe loss of function may therefore produce an experimentally less-informative phenotype (e.g., such as lethality). This approach has been successfully used to identify components of the *sevenless* (*sev*) RTK signal transduction pathway involved in the induction of the R7 fate during eye development (see e.g., Simon, et al., 1991. Cell 67:701-716).

The present invention comprises the use of *Drosophila* strains that are genetically-modified within the *Ras* proto-oncogene signaling pathway. A component of this pathway, the *Raf* serine/ threonine kinase, has been demonstrated to play a critical role in the signal transduction pathways activated by receptor tyrosine kinases (RTKs) across a broad phylogenetic spectrum. Within these signaling pathways, *Raf* acts to couple *Ras* activation to the mitogen-activated protein kinase (MAPK) cascade, which consists of the protein kinases MEK (MAPK kinase) and MAPK (see e.g., Marshall, 1995. Cell 80:179-185). The roles of these proteins in *Raf* signaling have been well-established by both biochemical and genetic studies. Less well-understood, however, are the roles of other *Raf*-binding proteins such as 14-3-3 proteins (see e.g., Fanti, et al., 1992. Nature 371:612-614; Freed, et al., 1994. Science 265:1713-1716; Fu, et al., 1994. Science 266:126-129), hsp90 (see e.g., Stancato, et al,. 1993. J. Biol. Chem. 268:21711-21716) and immunophilins (see e.g., Stancato, et al., 1994. J. Biol. Chem. 269:22157-22161), as well as the overall extent to which signal transduction via *Raf* is further regulated by as yet uncharacterized proteins acting within this, or parallel, signaling pathways.

In genetically modified *Drosophila* strains, specific signaling pathways involved in human diseases can be activated at a threshold to produce an easily detectable altered phenotype. It is preferred that such strains are genotypic (+/null), and hence are hemizygous for a dose sensitive gene within a given signaling pathway. These gene products are prime candidates as targets for small compounds that interfere with their function.

Activation of the Ras/MAP kinase cascade by Torso RTK results in the specification of head and tail regions in embryonic cells while activation of the same cascade by the sev RTK in the developing eye results in the specification of photoreceptor cell fate. It is possible that each of these receptors activates specific pathways in addition to the common Ras/MAP kinase pathway. The cell-type specific response may depend on which of these parallel pathway is activated by a given receptor. In vertebrate cell culture systems, it has for example been shown that the platelet derived growth factor (PDGF) receptor activates multiple signaling pathways. Identification of the general signaling components downstream of sev, namely Torso and DER (*Drosophila* homolog of the EGF (epidermal growth factor) receptor), is based on the study of loss-of-function (LOF) mutations, and indicate that the corresponding gene products thus identified are necessary for signaling. Indeed the complete removal of Drk, Sos, or Ras1 function in the Torso pathway produces a less severe phenotype than removal of either Torso or Raf function. This suggests that Torso can activate Raf independently of Drk, Sos and Ras1.

Additionally, *Drosophila* possessing the *rolled* gain of function mutation *Sevenmaker* (rl^{SEM}) display a number of additional phenotypes that resemble those of gain of function mutations in *torso* and *DER*. Embryos derived from rl^{SEM} females resemble those produced by females carrying a weak $torso^{GOF}$ mutation since they lack to a variable degree the central segmented region. Similarly, the formation of extra veins on the wing of rl^{SEM} flies is reminiscent of the Elp phenotype caused by a gain of function mutation in DER. Therefore, hyperactivation of MAP kinase is not only sufficient to activate the *sev* pathway but also the developmental pathways controlled by other RTKs.

The activation of RTK-specific signaling pathways that act in parallel to the general Ras/MAP kinase pathway is a possible way of maintaining the specificity of the inducing signal from the receptor to the nucleus. The decision of how a cell responds to the generic signal is taken in the nucleus and the nature of the response is determined to a large extent by the combination of nuclear factors present in the different cells at the time of induction. Hence, it may be an evolutionary advantage to use the same universal signaling cascade which can be activated by a number of different cell surface receptors to elicit a limited set of responses at any given stage in development and thereby successively restrict the developmental potential of cells.

It will be readily apparent to those individuals skilled in the art that other genetically-modified *Drosophila* strains may be used in the practice of the present invention. An important

attribute in choosing other appropriate *Drosophila* strains is that the strains have an easily monitored phenotype which is detectably-altered in response to the modification of genes related to disease pathways. Appropriate disease pathways include, but are not limited to, signaling pathways controlled by the *Ras* proto-oncogene; the *WNT* tumor suppressor gene; *Rb* (retinoblastoma tumor suppressor gene); *HH* (hedgehog development regulator) or the *HH* vertebrate homolog *SHH* (sonic hedgehog developmental regulator); activated protein kinase B (PKB/AKT); insulin receptor; insulin receptor substrates (IRS); *c-src* proto-oncogene; *c-Jun* proto-oncogene; *c-myc* proto-oncogene; p53; Janus kinases (JAK/STAT pathway); nitric oxide (NO); calmodulin; cAMP dependent protein kinase (PKA); Ca^{2+} dependent protein kinase (PKC); growth factors such as GH, TGF, PDGF and the like; receptor tyrosine kinases (RTKs); interferons (IFN); lipid metabolites; steroid hormones; phosphatidylinositol; G-protein coupled receptors; *c-abl* proto-oncogene; TGF-β and Smad gene family members; interleukins; GTPases; and ionophores. This list has been provided by way of example, for purposes of illustration only, and is not intended to be limiting with respect to scope, either real or implied.

The present invention discloses a methodology for the screening of genetically-modified *Drosophila* systems which are sensitive to gene dosage. The *Drosophila* phenotype which is to be assayed may involve the development of the eye, wing, or any other structure that develops from the imaginal discs in the fly. It should be noted that all previous assays to test the role of small molecular weight compounds have been performed with non-genetically-modified, wild-type *Drosophila*. Small molecular weight compounds will be administered by microinjection into the open circulatory system (i.e., hemolymph) of genetically-modified *Drosophila* first, second, or third instar larvae for subsequent use in high-throughput screening (HTS) assays of small molecular weight compound libraries and/or in the identification of the dose response curve (or other pharmacological parameters) of lead compounds of interest.

Any stage in *Drosophila* development may be used in the present invention. The *Drosophila* third instar larval stage may be chosen for both practical as well as specific considerations, which include, but are not limited to: (i) the third instar larvae are easily manipulated at this stage of development; (ii) the third instar larvae are large enough to facilitate automated microinjection in a high-throughput screening (HTS) assay; (iii) third instar larvae have an open circulatory system (i.e., hemolymph) through which there is rapid diffusion of the administered compound of interest; and (iv) in the third instar larvae myriad cellular signaling pathways are active in the growth and patterning of imaginal discs, which

give rise to the adult structure(s). At this stage any one of these pathways can be genetically sensitized in a way that small perturbation in its activity lead to readily detectable phenotypes in the adult. Perturbation may occur by mutations in genes coding for essential components or as in the case of this invention, by selecting small compounds on the basis of their ability to specifically interfere the adult phenotype. Of equal importance is the fact that the myriad cellular signaling pathways are extremely active during this stage of *Drosophila* development due to imaginal disc development.

As previously discussed, the present invention discloses the utilization of a genetically-modified *Drosophila* strain which is specifically modified such that it possesses a gene (within a specific signaling pathway known to be involved in human diseases) which can be activated at a defined threshold level to produce an easily-detectable phenotype (e.g., the generation of an irregular, rough eye surface phenotype). This genetic modification may be produced by a naturally-occurring, non-wild-type allele of a specific gene which is isolated from a genetic mutagenesis screening assay well-known to those individuals skilled within the art. Alternatively, the genetic modification may be produced by genetic manipulation using genetic recombination/molecular biological techniques known to those skilled within the art. One contemplated genetic modification is a non-wild-type allele which, when present in the heterozygous state, results in an altered phenotype that is dose dependent. As utilized herein, the term "dose dependent" is designated as meaning that the genetically-sensitized *Drosophila* strain exhibits an observably different phenotype for each specific genetic state when it possesses either none, one or two copies of the modified allele of the gene of interest.

The present invention utilizes the methodology of microinjection into the instar larval developmental stage of genetically-modified *Drosophila*, for example the third instar, thus permitting high-throughput screening (HTS) with subsequent automation of the assay procedure. This microinjection administration methodology offers several advantages over the conventional application by feeding. These advantages include, but not limited to: (i) both the time and developmental stage of the microinjection may be controlled in a precise manner; (ii) the amount, and thus the final concentration of the compound *in vivo* can be determined; (iii) the compound is rapidly dispersed through the open circulatory system and quickly reaches the target tissue, the imaginal discs; and (iv) the compounds are only administered at the time when the activity of the pathway is required to develop the easily scorable phenotype.

The HTS-based small molecular weight compound screening assay of the present invention is designed to ascertain (i.e., scores for) both the ability of the compound-treated larvae to undergo subsequent development (i.e., pupation) and for their eventual phenotype upon eclosion, in comparison to the mock-treated control *Drosophila* strain. The use of such *in vivo* assays allows for the highly relevant analysis of the bioavailability, biological/therapeutic function and toxicity of the compound(s) being tested. The present invention differs from conventional, HTS assays (based upon both cell-free or *in vitro* cell-based assay systems) in a number of ways including, but not limited to: (i) its overall degree of versatility; (ii) its indigenous screening capacity for compound specificity and non-toxicity and (iii) its lack of bias for specific classes of drug targets.

Without automation, the HTS-based small molecular weight compound screening assay of the present invention is capable of screening up to 100,000 compounds in a 1-2 month period. In contrast, with the use of an automated assay procedure, approximately 100,000 compounds may be screened in as little as 1-2 weeks. It should also be possible to scale up throughput with automated screening involving computer-assisted pattern recognition.

A wide variety of small molecular weight compounds may be used in the screen. Such compounds include, but are not limited to, any compositions which are being tested for lead drug discovery or development. Compounds may be aqueous- or lipid-soluble. Compounds may be delivered individually to separate *Drosophila* larvae or may be delivered to separate larvae as one of a plurality of different chemical compounds contained within a reagent solution, such as is performed within a multiplexing schema. Compounds may be dissolved or suspended within solution, or affixed to a solid-support. Solid supports may include, but are not limited to, insoluble polymer beads or a polymeric matrix coated with one or a plurality of individual compounds, or with combinatorial chemistries. Dosages and volumes which are microinjected into the *Drosophila* larvae may be varied so as to optimize dosages for further studies or to rank compounds as to their toxicity and/or potency. Information resulting from said variations in conditions may be used to prioritize chemicals for further study, to delineate the relative toxicities of structurally related chemicals, and/or to identify the proper dose range for subsequent toxicity studies (see e.g., Harris, et al., Fundam. Appl. Toxicol. 19:186-196).

In one embodiment of the present invention, recombinant DNA methodologies will be utilized to express exogenous genes which are functionally-linked to cell-specific transcriptional regulatory sequences. In an additional embodiment, exogenous genes which

encode human homologs of the genes involved in the signaling pathway of interest will be utilized, so as to enable "humanization" of the aforementioned disease pathways. An embodiment of the present invention involves the targeting of cell-specific expression of the incorporated exogenous genes to the cells of the *Drosophila* imaginal discs. Such genetic alterations possess the ability to greatly vary the genetic capabilities of the cells.

Compounds that are screened by use of the methodology disclosed in the present invention may be useful as analgesics and/or for the treatment of inflammatory disease, especially in the case of the azotricyclic compounds acting as antagonists of the neurokin 1/brandykin receptor. Members of the benzodiazopine library may be useful as a muscle relaxant and/or tranquilizer and/or as a sedative. Members of the 23 million Mixed Amide Library may be of use in the treatment of hypertension on endothelin antagonists or Raynaud's syndrome.

The carbon-carbon backbone of the compounds of the present invention may be saturated or unsaturated, cyclic or linear. These aforementioned compounds include, but are not limited to, carbohydrates, polyalcohols (e.g., ethylene glycol and glycerol) and polyphenols (e.g., hydroquinones and tetracylines). Carbohydrate- and polysaccharide-transformed compounds are defined herein so as to include all chemical moieties possessing a saccharide unit or which are transformed from a saccharide. These compounds may also include glycopeptides, glycolipids and other biopolymers (or biomacromolecules) containing saccharides, either in their entirety or as part of the molecular framework. The term carbohydrates merely represent a portion of a much larger family of polyhydroxylated organic compounds which are within the scope of the present invention. In addition, the carbohydrated/polyhydroxylated organic compounds of the present invention include, but are not limited to: monomeric acyclic compounds (e.g., ethylene glycol, glycerol and 1,2,3-trihydroxy pentane); polymeric acyclic compounds (e.g., di- or tri-ethylene diglycol; monomeric cyclic compounds (e.g., inositol and 1,2,3-trihydroxycyclopentane); polymeric cyclic compounds (e.g., di-inositol); polymeric and monomeric unsaturated compounds (e.g., tetrahydroxy-1,4-quinone) and polyphenols (e.g., tetracyclines) and derivatives, analogs and fragments thereof.

With respect to the generation of the small molecular weight compound libraries of the present invention, the combination of biochemical diversity is often synergistic with the metabolic diversity obtained from the *in vivo* production of "natural products". Collections of

starting compounds, for example peptides, can be administered to cultures of microorganisms. In accord, each microbial strain may potentially create numerous modified peptides or peptide byproducts, thus generating a "metabolite library". Because each of these aforementioned cultures would (potentially) contain a very complex mixture of metabolites, a highly efficacious method of screening would be required (i.e., HTS). An aliquot of the library is incubated with each of the many strains typical of a microorganism fermentation screening program, and the media screened utilizing an HTS-based assay. In another aspect of the invention, natural product diversity is screened by creating a mixture of combinatorially-tagged liposomes; wherein each liposome, for example, encapsulates only one member or a simple mixture of a natural product compound library. The libraries which are generated by the methodologies disclosed herein may be screened for any biological activity known within the art. These include, but are not limited to: anti-microbial activity, anti-tumor activity, enzyme inhibiting activity, receptor binding, growth promotion activity, and *in vitro* and *in vivo* tests for biological responses. Compounds may be based on naturally occurring extracellular or intracellular signaling molecules or their derivatives or the like (see, e.g., Alberts, et al., 1989. "Chapter 12: Cell Signaling." 2nd Edition. Garland Publishing, Inc., New York, NY, pp. 681-726).

Unfortunately, many peptidic-based compounds possess unfavorable pharmacodynamic properties such as poor oral bioavailability and rapid clearance *in vivo*, which have tended to limit the more widespread development of these compounds as potential therapeutic agents. This realization, however, has recently inspired workers to extend the concepts of combinatorial organic synthesis beyond peptide chemistry to create libraries of known pharmacophores (e.g., benzodiazepines; see e.g., Bunin, et al., 1992. J. Amer. Chem. Soc. 114:10997-10998), as well as polymeric molecules such as oligomeric N-substituted glycines (i.e., peptoids) and oligocarbamates (see e.g., Dower, et al., U.S. Patent No. 5,639,603).

The invention does not preclude the use of any type of compound library. Each library has its own specific advantages and disadvantages.

EXAMPLES

Example 1: Microinjection of Third Instar *Drosophila melanogaster* Larvae

Drosophila melanogaster which had been previously genetically-modified to activate a disease-related morphogenetic pathway in the developing compound eye are mated. Eggs are collected on a nylon mesh by use of standard techniques known within the art and placed onto

standard fly food. Approximately three to five day old larvae (third larval instar) are then collected and placed in the reservoir for the automated injection robot. Larvae are transported on a conveyer belt and immobilized through cooling to 10°C. Larvae are injected through the cuticle into the hemolymph with defined amounts of each compound using a hypodermic needle of 20 μm internal diameter.

Following injection, the larvae are placed into glass vials for completion of their development. After eclosion, the adult flies are anesthetized with CO_2 and visually inspected utilizing a dissecting microscope. The parameters which are scored include: (i) the overall toxicity of the compound (determined by the number of survivors compared to mock injected larvae); and (ii) the modification of the genetically-modified *Drosophila* phenotype. For example, in the case of the *raf* phenotype, the genetic modification which is scored is the suppression or enhancement of the irregular rough eye morphology.

Example 2: Generation of *Drosophila raf* Mutants

Su(Raf) and *E(Raf)* mutations were initially isolated by virtue of their ability to dominantly modify the rough eye phenotype in transgenic *Drosophila* carrying the activated *Raf* construct Raf^{torY9} (see e.g., Dickson, et al., 1996. Genetics 142:163-171). Figure 2 represents a schematic illustration of the known or estimated cytological chromosomal locations of all six *Su(Raf)* and two *E(Raf)* complementation groups (loci) (see e.g., Dickson, et al., 1996. Genetics 142:163-171). The Raf^{torY9} fusion produced was identical to that previously described (see e.g., Dickson, et al. 1992. Nature 360:600-603) with the exception that it contained the weakly-activating Y9 mutation in the *Torso* extracellular domain (see e.g., Sprenger & Nusslein-Volhard, 1992. Cell 71:987-1001). Two different Raf^{torY9} transgenic lines were used in this screening assay. The first was comprised of a second chromosomal insertion of a construct in which Raf^{torY9} is expressed under the control of a single *sev* enhancer and the heat-inducible *hsp70* promoter. This insertion is lethal in homozygotes. *Drosophila* carrying this chromosome balanced over the *CyO* balancer were used for the first rounds of mutagenesis and the Cy^+ progeny were scored. Subsequent rounds were performed using *Drosophila* homozygous for a chromosome 2 carrying a Raf^{torY9} fusion construct expressed under a duplicated *sev* enhancer and the *sev* promoter (see e.g., Dickson, et al., 1996. Genetics 142:163-171). The two Raf^{torY9} constructs were found to produced phenotypes of identical strength in heterozygotes. The first number of each allele indicated the round of mutagenesis in which the allele was isolated (i.e., numbers from 1-7 were isolated using the first construct and numbers

8-22 with the second construct). The *phyl* alleles 3G6 1 7L1 and 20Q1 have also been referred to as *phyl*^{1}, *phyl*^{2} and *phyl*^{3}, respectively (see e.g., Dickson, et al., 1995. Cell 80:453-462).

The first rounds (rounds 1-7) of mutagenesis were performed by mutagenizing w^{118} males isogenized for chromosome 2; whereas subsequent rounds (rounds 8-22) were performed with males reisogenized for the major autosomes. Males were treated with EMS (see e.g., Lewis & Bacher, 1968. *Drosophila* Info. Service 43:193-194) and crossed to *Raf*^{torY9} transformants. The efficiency of mutagenesis, as estimated by the induction of sex-linked lethal mutations, was consistently demonstrated to be on the order of ~0.60 lethal hits per major chromosomal arm.

Example 3: Isolation and Analysis of Modifiers of the *Raf*^{torY9} Phenotype by Genetic Means

The following example shows the method by which genes that modify a given pathway of choice can be identified using genetic means. In the manner of the invention described *supra*, corresponding small molecular weight compounds that modify a given pathway of choice will be used with the resulting genetically modified *Drosophila* strains to identify biologically active "lead" compounds.

The Raf kinase may be activated either by truncation of its amino-terminal domain (see e.g., Stanton, al., 1989. Mol. Cell Biol. 9:639-647) or by relocation of the entire protein to the cell membrane following post-translational modification at an artificial carboxyl-terminal CAAX site (see e.g., Leevers, et al., 1994. Nature 369:411-414; Stokoe, et al., 1994. Science 264:1463-1467). Similarly, the *Drosophila* Raf kinase is activated following amino-terminal truncation and relocation to the cell membrane via fusion to the extracellular and transmembrane domains of the Torso protein, a receptor tyrosine kinase (RTK) (see e.g., Dickson, et al. 1992. Nature 360:600-603). It should be noted that the Raf kinase activity of this aforementioned fusion protein may be further increased by introducing point mutations in the Torso extracellular domain which are analogous to dominantly activating mutations in the Torso RTK, tor^{Y9} and tor^{4021} (see e.g., Sprenger & Nusslein-Volhard, 1992. Cell 71:987-1001).

Transgenic *Drosophila* possessing the activated Raf construct *Raf*^{torY9} expressed under the transcriptional control of an enhancer element of the *sevenless* (*sev*) gene were generated (see e.g., Basler, et al., 1991. Cell 64:1069-1081). In these transgenic Drosophila, the Raf kinase was found to be constitutively activated in the five cells of the developing eye which

choose between the alternative fates of development as an R7 photoreceptor or a non-neuronal cone cell. Typically, Raf is activated in only one of these five cells (i.e., the R7 precursor) via the local activation of the sev RTK. Hence, activation of Raf is both necessary and sufficient for this cell type to select the R7 fate (see e.g., Dickson, et al., 1992. Genetics 142:163-171). In *Raf*^{torY9} *Drosophila* mutants, ectopic Raf activity within the cone cell precursors results in some of these cells also developing as R7 cells. As a result, most of the 800 ommatidia found within the *Drosophila* compound eye were found to contain several, instead of just one, R7 photoreceptors. This result is illustrated in Figure 1 (panel F) and these abnormal ommatidia disrupt the regular hexagonal ommatidial lattice. The *Drosophila* eye thus acquires a "roughened" external appearance, readily observed in live, anaesthetized flies (see Figure 1, panel B). The degree of roughening generally reflects the average number of additional R7 cells per ommatidium, and thus provides an indirect measure of the efficiency of Raf signaling within the cone cell precursors. This observation would thus allow for the identification of "lead" compounds which dominantly-modify the degree of roughening and thus potentially affect some step within the cellular signaling pathway between *Raf* activation and the selection of the R7 fate.

Example 4: Interactions with a Hypomorphic *raf* Allele

The following example shows the method by which genes that modify a given pathway of choice can be identified using genetic means. In the manner of the invention described *supra*, corresponding small molecular weight compounds that modify a given pathway of choice will be used with the resulting genetically modified *Drosophila* strains to identify biologically active "lead" compounds.

A complementary screen was performed in which mutations that dominantly-modify the phenotype of a gain-of-function (GOF) *raf* allele affecting only R7 cell development were isolated. The primary advantage of this approach is that, by specifically-addressing R7 development, it was possible to avoid complications with the formation of lethal genetic *Drosophila* mutations. Additionally, this type of approach also permitted simultaneously screening for both suppressor *Su(Raf)* and enhancer E(*Raf*) mutations. Thus, both positive and negative regulators may potentially be identified with a far greater degree of efficiency by the recovery of LOF alleles.

The gain-of-function (GOF) construct *Raf*^{torY9}, provided a convenient means for isolating interacting mutations. There is some evidence, however, that these aforementioned mutations may interact specifically with this construct (e.g., with either the *sev* enhancer or *Torso* domain) and not with the *Raf* kinase itself. It was therefore necessary to test each *Su(Raf)* and E(*Raf*) locus for genetic interactions with the hypomorphic *raf* allele, *raf*^{HM7}. The *raf*^{HM7} allele produces reduced levels of the wild-type protein, sufficient for *Drosophila* survival at 18°C but not at 25°C (see e.g., Melnick, et al., 1993. Development 118:127-138). Raised at the permissive temperature of 18°C, only 50% of ommatidia in the eyes of hemizygous *raf*^{HM7} *Drosophila* males were found to contain an R7 cell (see e.g., Dickson, et al., 1992. Nature 360:600-603).

Various result scenarios are possible. For example, if it was demonstrated that the *Su(Raf)* mutations impaired signaling via *Raf*, it would be expected that they would enhance the *raf*^{HM7} phenotype. If it was found that the *Su(Raf)* mutations are generally required for *Raf* function, this may result in a synthetic lethality (i.e., non-allelic, non-complementation) at 18°C. On the other hand, if the *Su(Raf)* mutations were demonstrated to be specifically required for *Raf* function within the *Drosophila* eye, it would be expected that they would enhance the *raf*^{HM7} eye phenotype without affecting the viability of these flies. Conversely, E(*Raf*) mutations, if they were demonstrated to relieve negative influences on *Raf* signaling, it would be expected that they would suppress the *raf*^{HM7} phenotype.

Table 1, entitled "Genetic interactions with *raf*^{HM7}", illustrates the genetic interactions between *raf*^{HM7} and *Su(Raf)* and two E(*Raf*) loci. For viable combinations following mating, the eyes of both classes were compared for any modification of the mild degree of roughening caused by the *raf*^{HM7} mutation, alone. Four of the *Su(Raf)* loci were found to enhance the *raf*^{HM7} phenotype in accordance with these aforementioned predictions. Of these loci, three (*rl*, *Su(Raf)*3A and *Su(Raf)*3B) are synthetically lethal in combination with *raf*^{HM7}, thus suggesting that they encode proteins generally required for signal transduction via *Raf*. In agreement with this prediction, *rl* has also been shown to encode a MAPK homologue required in several signaling events involving *Raf* (see e.g., Biggs, et al., 1994. EMBO J. 13:1628-1635; Brunner, et al., 1994. Nature 370:386-389). The fourth of these loci, *phyl*, enhances only the eye phenotype of *raf*^{HM7}, which was demonstrated to significantly increases the number of ommatidia lacking R7 cells (see e.g., Dickson, et al., 1995. Genes Dev. 6:2327-2339). This finding is consistent with the interpretation disclosed herein of *phyl* as a target gene

transcriptionally-regulated only within R7 cells and two other photoreceptors, in response to activation of the *Raf* pathway. It should be noted that, at present, there is no evidence for *phyl* acting as a target gene for *Raf* signaling in any other tissue. The other two *Su*(*Raf*) loci - *Su*(*Raf*)2A and *Su*(*Raf*)3C - show no dominant genetic interaction with raf^{HM7}.

Somewhat surprisingly, neither of the E(*Raf*) loci were found to suppresses raf^{HM7}, but rather enhance the hypomorphic *raf* phenotype, resulting in synthetic lethality. The E(*Raf*)2A allele 1401 appears to be weaker than the other two alleles at this locus, and a small number of flies of the genotype raf^{HM7}/Y; 1401/+ eclose. Additionally, the raf^{HM7} eye phenotype is also slightly enhanced in these flies. Since the E(*Raf*) mutations enhance the phenotypes of both LOF and GOF *raf* alleles, it is considered to be unlikely that these genetic interactions reflect direct biochemical interactions between the proteins these genes encode and *Raf*.

Genetic interactions with raf^{HM7}

Locus	Allele tested	No. of raf^{HM7}; *Su*/+ or raf^{HM7}; E/+ males	No. of raf^{HM7}; Pw^{+}/+ males	Relative viability (%)	Modification of raf^{HM7} eye phenotype
Su(Raf)2A	*3E8*	73	136	54	None
	4P5	54	45	120	None
rolled	*2L1*	0	61	0	—
	6L1	0	63	0	—
phyllopod	*3G6*	50	53	94	Enhanced
	17L1	156	118	132	Enhanced
Su(Raf)3A	*9J1*	0	83	0	—
	19F2	0	60	0	—
Su(Raf)3B	*18A2*	0	174	0	—
Su(Raf)3C	*17M1*	127	121	105	None
E(Raf)2A	*14O1*	13	72	18	Enhanced
	16H1	0	135	0	—
	16T1	0	157	0	—
Star	*2J1*	0	50	0	—

Genetic interactions between raf^{HM7} and *Su(Raf)* and *E(Raf)* loci. raf^{HM7}, w^{a}/*FM7* virgins were mated to *Su(Raf)*/Pw^{+} or *E(Raf)*/Pw^{+} males, and the total number of raf^{HM7} progeny surviving to adulthood were scored for each class. For viable combinations, the eyes of both classes were compared for any modification of the mild roughening caused by the raf^{HM7} mutation.

Example 5: Homozygous *Su(Raf)* and E(*Raf*) Phenotypes

The following example shows the method by which genes that modify a given pathway of choice can be identified using genetic means. In the manner of the invention described *supra*, corresponding small molecular weight compounds that modify a given pathway of choice will be used with the resulting genetically modified *Drosophila* strains to identify biologically active "lead" compounds.

The recessive phenotypes of *rl*, *phyl* and *Star* mutations have been reported elsewhere (see e.g., Heberlein, et al., 1993. Dev. Biol. 144:353-361; Biggs, et al., 1994. EMBO J. 13:1628-1635; Chang, et al., 1995. Cell 80:463-472; Dickson, et al., 1995. Genes Dev. 6:2327-2339). The *rl* phenotype appears to be required for the development of all eight photoreceptors; whereas *phyl* is required specifically for R1, R6 and R7. However, there is only circumstantial evidence for a role of *Star* in R7 development (see e.g., Kolodkin, et al., 1994. Development 120:1731-1745), but in any case the recovery of a *Star* allele in our screen is more likely due to its dominant rough eye phenotype than a specific interaction with Raf^{torY9}.

Surprisingly, three of the *Su(Raf)* genes do not appear to be absolutely required for development either of R7 or any other photoreceptor. The single *Su(Raf)*3B and *Su(Raf)*3C alleles are both viable in the homozygous condition and show no obvious defects in eye development. *Su(Raf)*2A mutations are homozygous lethal, but patches of homozygous mutant tissue could readily be recovered in heterozygous animals using the FLP/FRT technique to induce site-specific mitotic recombination (see Xu & Rubin, 1993. Development 117:1223-1237). Examination of such homozygous *Su(Raf)*2A clones shows that, although required for viability, this gene is dispensable for normal *Drosophila* eye development.

*Su(Raf)*3A mutations were also demonstrated to be lethal in the homozygous state, and it proved to be impossible to generate *Su(Raf)*3A mutant clones by mitotic recombination by use of the methodologies of the present invention.

This suggests that *Su(Raf)*3A, like *raf* and *rl*, may also be required for cell proliferation (see e.g., Nishida, et al., 1988. EMBO J. 7:775-781; Biggs, et al., 1994. EMBO J. 13:1628-1635). The requirement of *Su(Raf)*3A in the early proliferative phase of eye development precludes a direct examination of its role in the later stages of eye development during which cell fates are determined.

In conclusion, E(*Raf*)2A has been demonstrated to be required for both viability and eye development Homozygous mutant ommatidia were found to be of variable composition, often lacking either R7 or one or more other photoreceptors, but also occasionally containing extra photoreceptors of either class.

Example 6: *Su(Raf)*2A is Required for Ectopic R7 Development

The following example shows the method by which genes that modify a given pathway of choice can be identified using genetic means. In the manner of the invention described *supra*, corresponding small molecular weight compounds that modify a given pathway of choice will be used with the resulting genetically modified *Drosophila* strains to identify biologically active "lead" compounds.

Interestingly, it was found that the elimination of one copy of *Su(Raf)*2A severely impairs signaling via Raf^{torY9}; whereas elimination of both copies of the gene did not appear to affect signaling via the endogenous Raf kinase. One possible explanation of this result would be that *Su(Raf)*2A encodes a protein that interacts specifically with the activated Raf^{torY9} fusion protein but not at all with the endogenous Raf. To test this hypothesis, *Su(Raf)*2A clones were generated in genetic backgrounds in which the *Raf* pathway is constitutively activated at points further upstream, i.e., at *Sev* in *Sev*^{S11} transformants; (see e.g., Basler, et al., 1991. Cell 64:1069-1082) and at *Ras*1 in *Ras*1 V12 transformants (see e.g., Fortini, et al., 1992. Nature 355:559-561). This aforementioned assay resulted in the formation of ectopic, but not endogenous R7 cells is completely blocked within the *Su(Raf)*2A clone. This observation argues strongly against a specific interaction between Su(Raf)2A and Raf^{torY9} and suggests that the protein encoded by *Su(Raf)*2A is indeed important for signaling via the wild-type Raf kinase, at least for the generation of ectopic R7 cells due to constitutive activation of the pathway at or above Raf.

Ectopic R7 cells are also formed in *yan* mutants in an independent manner from the *Raf* pathway (see e.g., Lai, et al., 1992. Cell 70:609-620). It is believed that the Raf/MAPK pathway acts in part to overcome the inhibitory influence on R7 development normally exerted via the Yan protein (see e.g., Brunner, et al., 1994. Nature 370:386-389; O'Neill, et al., 1994. Cell 78:137-147; Rebay, et al., Cell 81:857-866). The formation of extra R7 cells in *yan* mutants is also independent of Su(Raf)2A, thus suggesting that in *Su(Raf)*2A mutant tissue the pathway leading to ectopic R7 development is blocked at some point between Raf and Yan.

Example 7: Injection of Rapamycin as a Demonstrative Example

To demonstrate the effectiveness of the injection system we developed a protocol for small scale experiments. As a model we used *Drosophila* larvae genetically modified to express constitutively active PI3 kinase (PI3K) in the compound eye, and Rapamycin, a well known inhibitor *in vitro* and *in vivo* of the TOR kinase. Both PI3K and TOR are downstream components of the insulin receptor regulated growth pathway. As a result of the over-activation of this pathway, the eyes of these flies, called R10, are bigger and more irregularly shaped than wild type eyes.

Early phase third instar larvae were anaesthetized for 2 minutes with ether and injected manually with increasing amounts of either a 50 μM solution of Rapamycin in DMSO (dark bars) or DMSO solvent only (light bars) using an Eppendorf microinjector. The injected larvae were transferred to culture tubes and allow to develop at 25°C to adult flies. Flies were then scored for phenotypic changes under the microscope.

As shown in FIG. 3, 70 % of the larvae survived the injection procedure, with an injection mortality of about 20%. However, Rapamycin caused a high mortality even at quite low dosages. Larval survival was dose-dependent and reproducible. In addition, 100-500 pi resulted in a significant rapamycin effect (survival and size) without significant unspecific toxicity. R10 flies surviving the injection normally had eyes that were significantly smaller in 80% of rapamycin-injected flies. At higher injection doses only, flies treated with 100-500 pi were also up to 30% smaller than control injected flies. Therefore, the specific reversion of the big eye phenotype at lower dosages demonstrates that genetically modified Drosophila larvae can be used to screen for specific inhibitors of a particular biochemical pathway.

EQUIVALENTS

Specific embodiments have been exemplified herein for purposes of illustration only, and are not intended to be limiting with respect to the scope of the appended claims. In particular, various substitutions, alterations, and/or modifications may be made to the invention without departing from the spirit and scope of the invention as defined by the claims.

WHAT IS CLAIMED IS:

1. A method of using genetically-modified *Drosophila* strains in a high-throughput screening assay to screen for compounds putatively affecting a biochemical pathway involving said genetic modification whose activity or function is critical for the development of a phenotype and which can be activated so as to generate an easily detectable phenotype within said genetically-modified *Drosophila* strains..

2. The method of claim 1, wherein said genetic modification of *Drosophila* is comprised of modification of a chromosomal sequence which is related to, and functions in, a disease system or pathway.

3. The method of claim 1, wherein said disease system or pathway is a human disease system or pathway.

4. A method of using a larva from a chosen genetically-modified *Drosophila* strain for high-throughput screening of one or more compounds which putatively affect the biochemical pathway involving said genetic modification, whereby said genetic modification involves the development of an imaginal disk into its mature structure during pupation; comprised of the following steps:

(a) microinjecting at least one compound, alone or in combination, into the open circulatory system (hemolymph) of a genetically-modified *Drosophila* larva;

(b) allowing said microinjected genetically-modified *Drosophila* larva to undergo pupation; and

(c) analyzing the assay results by interpretation of the physiological or morphological effect of said microinjected compound on the insect's adult structure formed from the imaginal disk as seen in the *Drosophila* imago following eclosion of the mature imago from the pupa.

5. The method of claim 4, where in the genetically-modified *Drosophila* larva is chosen from a first, second, or third instar larva.

6. The method of claim 4, where in the *Drosophila* larva is a third instar larva.

7. The method of claim 4, wherein the insect's adult structure formed from the imaginal disk is the compound eye.

8. The method of claim 4, wherein said physiological or morphological effect is the effect of said microinjection compound on the irregular rough eye phenotype of the *Drosophila* compound eye.

9. The method of claim 4, wherein said compounds are comprised of small molecular weight compounds.

10. The method of claim 4, wherein said compounds, which may be natural products, non-natural products or combinations thereof, are selected from the group consisting of: amino acids, polypeptides, proteins, nucleotides, carbohydrates, lipids, steroids, coenzymes, enzyme inhibitors, ligands and pharmaceuticals such as anti-hypertensive agents, anti-ulcer drugs/H2-receptor antagonists, anti-fungal agents, cholesterol-demethylase inhibitors, anxiolytic agents, analgesics, antibiotics, anti-inflammatory agents, contraceptives, abortifacients, anti-histamines, anti-tussive agents and sedatives, as well as modifications, derivatives and analogs thereof.

11. The method of claim 4, wherein said compounds are attached to an insoluble, solid support matrix by linkages which are cleavable by alteration of the physical environment of said matrix.

12. The method of claim 4, wherein said genetic modification of *Drosophila* is comprised of modification of a chromosomal sequence which is related to, and functions in, a disease system or pathway.

13. The method of claim 12, wherein said disease system or pathway is a human disease system or pathway.

14. The method of claim 4, wherein said genetic modification of *Drosophila* is comprised of modification of one or more gene(s) selected from the group comprising: a *Raf* gene; a *Ras* proto-oncogene system or pathway; a *WNT* tumor suppressor system or pathway; a hedgehog development regulator (*HH*) system or pathway; a sonic hedgehog development regulator (*SHH*) system or pathway; a retinoblastoma tumor suppressor (*Rb*) system or pathway; an activated *Drosophila* protein kinase B (PKB/AKT) gene; an activated human PKB/AKT gene; an activated insulin receptor gene; an insulin receptor substrates (IRS) gene or pathway; *c-src* proto-oncogene or pathway; *c-Jun* proto-oncogene or pathway; *c-myc* proto-oncogene or pathway; p53; Janus kinases (JAK/STAT pathway); nitric oxide (NO); calmodulin; cAMP dependent protein kinase (PKA) or pathway; Ca^{2+} dependent protein kinase (PKC) or pathway; growth factors such as GH, TGF, PDGF and the like; receptor tyrosine kinases (RTKs) and pathways; interferons (IFN) or pathways; lipid metabolites or pathway; steroid hormones or pathway; phosphatidylinositol or pathway; G-protein coupled receptors or pathways; *c-abl* proto-oncogene or pathway; TGF-β and Smad gene family members; interleukins or pathway; GTPases or pathway; and ionophores or pathway.

15. A genetically modified *Drosophila* strain expressing one or more native or modified genes selected from the group comprising: a *Raf* gene; a *Ras* proto-oncogene system or pathway; a *WNT* tumor suppressor system or pathway; a hedgehog development regulator (*HH*) system or pathway; a sonic hedgehog development regulator (*SHH*) system or pathway; a retinoblastoma tumor suppressor (*Rb*) system or pathway; an activated *Drosophila* protein kinase B (PKB/AKT) gene; an activated human PKB/AKT gene; an activated insulin receptor gene; an insulin receptor substrates (IRS) gene or pathway; *c-src* proto-oncogene or pathway; *c-Jun* proto-oncogene or pathway; *c-myc* proto-oncogene or pathway; p53; Janus kinases (JAK/STAT pathway); nitric oxide (NO); calmodulin; cAMP dependent protein kinase (PKA) or pathway; Ca^{2+} dependent protein kinase (PKC) or pathway; growth factors such as GH, TGF, PDGF and the like; receptor tyrosine kinases (RTKs) and pathways; interferons (IFN) or pathways; lipid metabolites or pathway; steroid hormones or pathway; phosphatidylinositol or pathway; G-protein coupled receptors or pathways; *c-abl* proto-oncogene or pathway; TGF-β and Smad gene family members; interleukins or pathway; GTPases or pathway; and ionophores or pathway.

16. The method of claim 4, wherein said microinjection procedure is automated.

17. The method of claim 4, wherein said high-throughput screening procedure is automated.

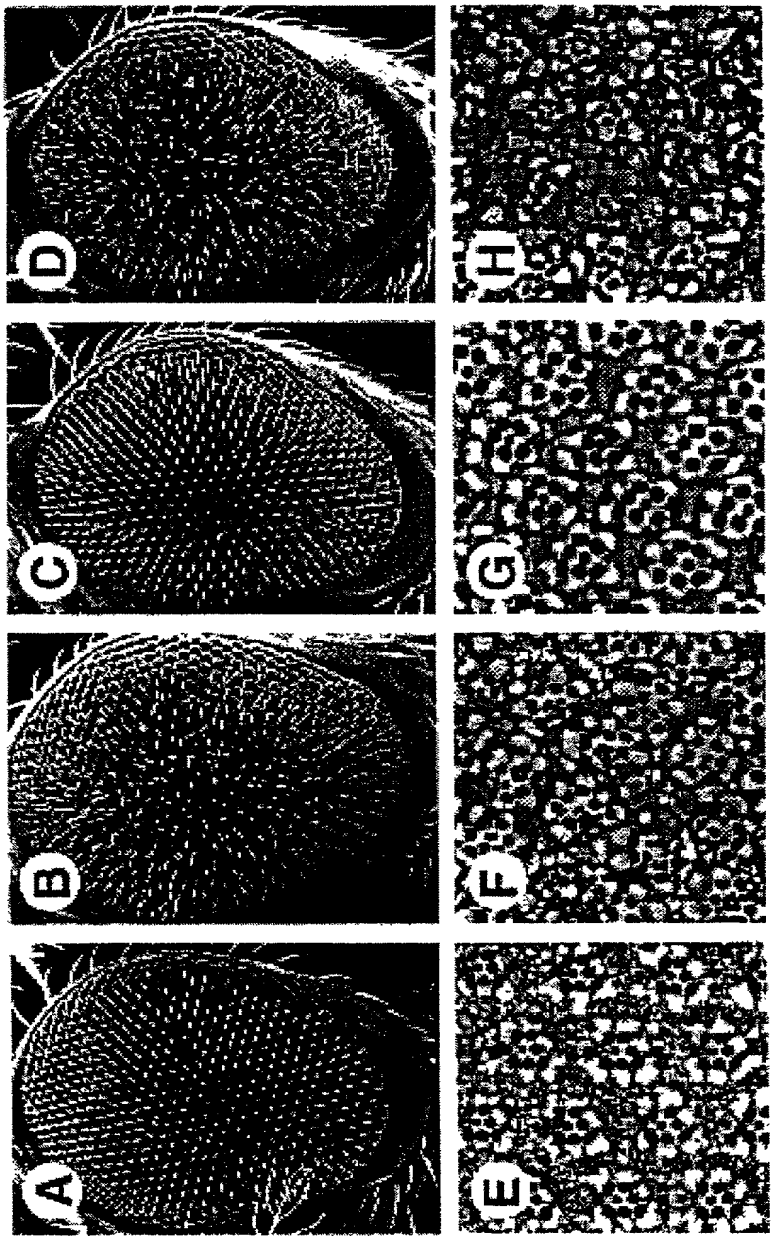
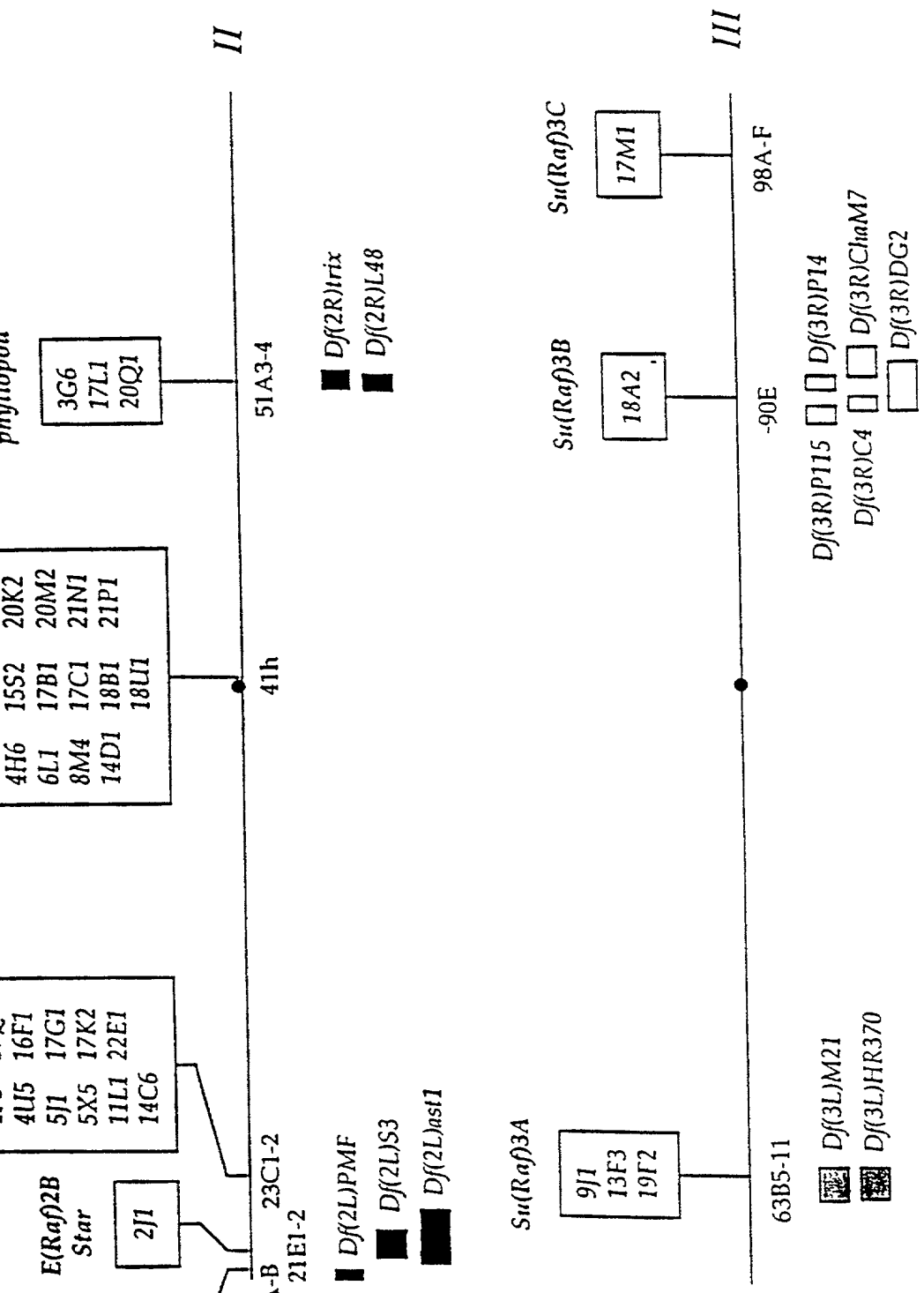


FIG. 1


II
III
Su(Raf)2B
rolled
2L1 14D5 19E1
2M1 15M1 19U1
4H6 15S2 20K2
6L1 17B1 20M2
8M4 17C1 21N1
14D1 18B1 21P1
18U1
Su(Raf)2C
phyllopod
3G6
17L1
20Q1
51A3-4
41h
Df(2R)Irix
Df(2R)L48
Su(Raf)2A
3E8 15D1
4P5 16Q1
4U5 16F1
5J1 17G1
5X5 17K2
11L1 22E1
14C6
E(Raf)2B
Star
2J1
E(Raf)2A
14O1
16H1
16T1
21A-B 23C1-2
21E1-2
Df(2L)PMF
Df(2L)S3
Df(2L)ast1
Su(Raf)3A
9J1
13F3
19F2
63B5-11
Df(3L)M21
Df(3L)HR370
Su(Raf)3B
18A2
-90E
Su(Raf)3C
17M1
98A-F
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Df(3R)P14
Df(3R)C4
Df(3R)ChaM7
Df(3R)DG2

FIG. 2

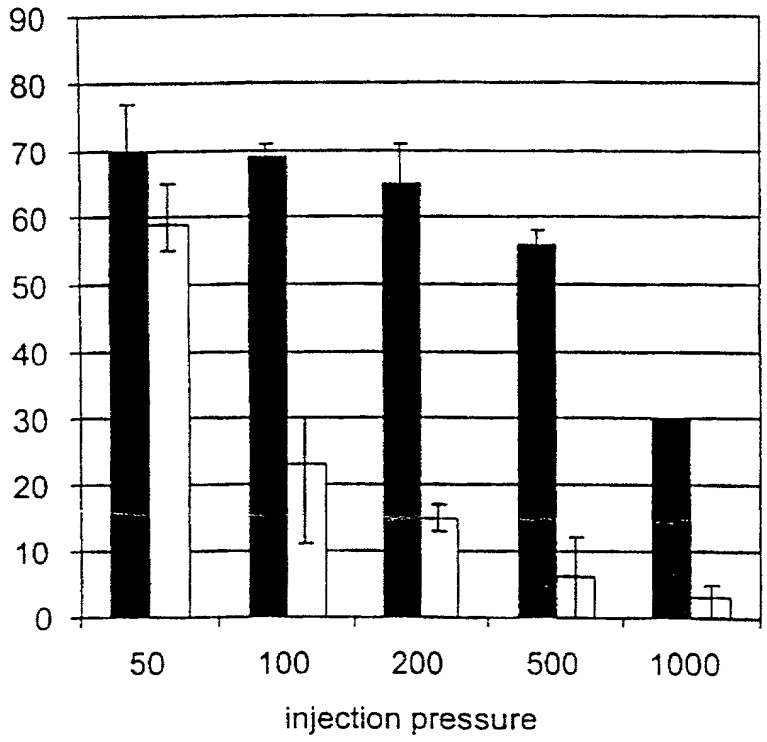
90
80
70
60
50
40
30
20
10
0
50
100
200
500
1000
injection pressure

FIG. 3

Express Mail No.:
Date of Deposit:

Attorney Docket No.: 18111-001 NATL

COMBINED DECLARATION AND POWER OF ATTORNEY FOR PATENT APPLICATION

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am an original, first and sole inventor of the subject matter which is claimed and for which a utility patent is sought on the invention entitled:

FUNCTION-BASED SMALL MOLECULAR WEIGHT COMPOUND SCREENING SYSTEM IN *DROSOPHILA MELANOGASTER*

the specification of which:

☒ was filed on **16 December 1999**, as PCT application **PCT/IB99/02020**, and Nationalized in the United States as Serial No. **09/868,748**, bearing Attorney Docket No. 18111-001 NATL.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information that is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56.

☒ I hereby claim the benefit under Title 35, United States Code, § 119(e) or §120 of any United States application(s), or §365(c) of any PCT International application(s) designating the United States of America listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application:

Application No. *(U.S.S.N.)*	**Filing Date** *(dd/mm/yy)*	**Status** *(Patented, Pending, Abandoned)*
09/217,694	21/12/98	Pending

PCT International Applications designating the United States:

PCT Appln No.	US Serial No.	PCT Filing Date	Status
PCT/IB99/02020		16/12/99	

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or patent issued thereon.

Jan 12, 2002

Signature of Ernst Hafen Date

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